RESEARCH HIGHLIGHT

# Asynchronous inflammation and myogenic cell migration limit muscle tissue regeneration mediated by a cellular scaffolds

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**Volumetric muscle loss (VML) following orthopaedic trauma results in chronic loss of strength and can contribute to disability. Tissue engineering and regenerative medicine approaches to regenerate the lost skeletal muscle and improve functional outcomes are currently under development. At the forefront of these efforts, decellularized extracellular matrices (ECMs) have reached clinical testing and provide the foundation for other approaches that include stem/progenitor cell delivery. ECMs have been demonstrated to possess many qualities to initiate regeneration, to include stem cell chemotaxis and pro-regenerative macrophage polarization. However, the majority of observations indicate that ECM-repair of VML does not promote appreciable muscle fiber regeneration. In a recent study, ECM-repair of VML was compared to *classical* muscle fiber regeneration (Garg *et al*., 2014, Cell & Tissue Research) mediated by autologous minced grafts. The most salient findings of this study were: 1) Satellite cells did not migrate into the scaffold beyond ~0.5 mm from the remaining host tissue, although other migratory stem cells (Sca-1$^{+}$) were observed throughout the scaffold;2) Macrophage migration to the scaffold was over two-times that observed with muscle grafts, but they appeared to be less active, as gene expression of pro- and anti-inflammatory cytokines (TNF-α, IL-12, IL-4, IL-10, VEGF, and TGF-ß1) was significantly reduced in scaffold-repaired muscles; And, 3) scaffolds did not promote appreciable muscle fiber regeneration. Collectively, these data suggest that the events following ECM transplantation in VML are either incongruous or asynchronous with *classical* muscle fiber regeneration.**

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Orthopaedic trauma in civilian and military populations usually involves severe skeletal muscle injury. Either directly (e.g., blast injury) or indirectly (e.g., soft tissue evacuation secondary to crush), the traumatized musculature often presents volumetric muscle loss (VML) [1]; which mammalian skeletal muscle is not capable of successfully regenerating [2-4]. There is currently no effective therapy used in the clinic that promotes *de novo* muscle tissue regeneration following VML. To this end, persistent strength deficits and limb dysfunction are currently anticipated as part of the sequelae of this injury [5]. Multiple efforts are ongoing to develop tissue engineering and regenerative medicine therapies for the repair of VML- the intention of the therapies is to restore strength to the injured musculature by regenerating appreciable and functional muscle tissue. At the forefront of these efforts are decellularized extracellular matrix scaffolds (ECMs) (**Table 1**), which are being investigated in the clinic for VML [6]. While ECMs represent a logical therapy for VML, by-and-large the capacity of ECMs to promote *de novo* skeletal muscle regeneration appears to be limited [3, 6-8]. The purpose of this report is to compare the putative mechanism of ECM-mediated regeneration with that of classical muscle repair and regeneration.

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**Table 1. Selected studies of VML repair (Listed chronologically)**

| | VML Model | VML Size | Species | Therapy | Primary Histological Outcome |
|---|---|---|---|---|---|
| Brown et al.[8] | Abdominal Wall | 1cm x 1cm | Rats | Rat Muscle Autograft | Fibrosis and inflammation |
| | | | | Rat mECM | Fibrosis and small islands of myofibers |
| | | | | Porcine Muscle Graft | Fibrosis with inflammation |
| | | | | Porcine UBM | Fibrosis and small islands of myofibers |
| Turner et al. [36] | Gastrocnemius | N/A | Dogs | Porcine SIS | Myofiber regeneration throughout defect area |
| Merritt et al. [13, 49] | Gastrocnemius | ~225 mg | Rats | Rat mECM | Limited ingrowth of myofibers |
| | | | | Rat mECM + MSCs | Improved but limited myofiber regeneration |
| Mase et al. [4] | Quadriceps | N/A | Human | Porcine SIS | N/A : CT showed unknown tissue deposition |
| Machingal et al. [50] | Latissimus dorsi | ~ 25 mg | Mice | Porcine BAM + MDCs | Desmin+ cells throughout defect & fibrosis |
| | | | | Porcine BAM | Fibrosis & diminished myofiber regeneration |
| Sicari et al. [14] | Quadriceps | 4 x 4 x 3 mm | Mice | Porcine SIS | Fibrosis & small islands of myofibers |
| Corona et al. [3] | Tibialis anterior | 10x7x3 mm | Rats | Rat mECM | Fibrosis with limited muscle regeneration |
| | | | | Rat mECM + MSCs | Fibrosis with limited muscle regeneration |
| Corona et al. [2] | Tibialis anterior | 10x7x3 mm | Rats | Rat Minced Muscle Grafts | Myofiber regeneration throughout defect area |
| Sicari et al. [6] | Quadriceps | ~ 75% loss | Mice | Porcine UBM | Fibrosis & limited ingrowth of muscle fibers |
| | Lower Extremity | N/A | Human | Porcine UBM | Fibrosis & small islands of myofibers |
| Ma et al. [12] | Abdominal Wall | 1.2 x 1.2 cm | Rat | Porcine SIS | Fibrosis with no muscle fiber regeneration |
| Garg et al. [16] | Tibialis Anterior | 10x7x3 mm | Rat | Rat Minced Muscle Grafts | Myofiber regeneration throughout defect area |
| | | | | Rat Muscle Scaffold | Fibrosis throughout defect area |

mECM (muscle ECM); UBM (urinary bladder matrix ECM); SIS (small intestine submucosa ECM); MSC (mesenchymal stem cell); CT (computed tomography); BAM (bladder acellular matrix ECM); MDCs (muscle-derived cells)

The precise mechanism underlying ECM-mediated regeneration in skeletal muscle has not been completely elucidated. Following transplantation in a fresh VML wound bed, ECMs are infiltrated with mononuclear cells within hours [9]. Thereafter, ECMs substantially degrade over the ensuing month [10, 11]. Within this time, ECMs are thought to promote skeletal muscle regeneration *in vivo* by orchestrating the following events through an undefined spatiotemporal pattern (*see review:* [9]): **1**) promotion of a vascular bed, **2**) chemotaxis for nearby resident and circulating progenitor and stem cells,**3**) provision of matrix bound growth factors and cryptic peptides that direct functions of migrating cells, and **4**) induction of pro-regenerative macrophage polarization *in vitro*. Ultimately, it is thought that the degrading ECM creates a pro-regenerative environment that promotes myogenic differentiation of migrating stem and progenitor cells, which then effectively regenerate functional muscle fibers.

Despite the regenerative attributes of ECMs, many studies present evidence at prolonged time points (months) that ECMs do not promote appreciable muscle fiber regeneration *in vivo*, particularly in regions of the defect further removed from the remaining tissue bed- instead fibrotic tissue is remodeled [3, 6, 7, 12-15]. These observations have been made among various labs in different species and VML models using a variety of ECMs derived from autologous, syngeneic, or xenogeneic tissues, to include bladder, small intestine, and muscle (**Table 1**). That being said, fibrotic tissue deposition following ECM transplantation may have under-appreciated therapeutic benefits, to include improving strength via improved force transmission (as opposed to production) [3] and protecting the remaining muscle mass from chronic overload-induced injury [2, 3].

Recently we observed restricted and limited muscle fiber regeneration after autologous devitalized muscle scaffold repair of VML in the rat TA muscle two months post-injury [16]. Given this fairly consistent observation (**Table 1**), we posited that while ECM preparation may explain subtle variations in regenerative outcomes [17], it is more likely that the underlying mechanism of ECM-remodeling in a VML defect does not adequately mimic that of adult endogenous adult muscle fiber regeneration. To investigate this possibility, we further interrogated at an acute time point (2 weeks post-injury) the inflammatory and myogenic response to the muscle scaffold [16], which relies solely on host cell migration for regeneration [18]. Vital minced grafts (1 $mm^3$ pieces of tissue), which also deliver satellite cells among other cellular and trophic factors resident in whole skeletal muscle, were used as a positive control of successful muscle regeneration[2, 18-20]. In the following sections, the findings of this study are highlighted and discussed in the context of events necessary for classical muscle repair and regeneration.

Satellite cells ($Pax7^+$) are a skeletal muscle specific stem cell population that is indispensable for muscle regeneration following injury [21]. A combination of signals from the host muscle fibers, circulating cells, interstitial cells (such as macrophages) and muscle resident stem cells can influence

the quiescence, activation and proliferation of the satellite cells [22]. It has been shown that, over 100 new myofibers containing thousands of myonuclei can be generated from as little as seven activated satellite cells associated with one transplanted myofiber [23]. The loss of satellite cell pool due to aging or disease (such as duchenne muscular dystrophy) results in impaired regeneration, increased atrophy and fibrosis of skeletal muscle [24-27]. It has also been reported that genetic elimination of satellite cells results in a complete blockade of regenerative myogenesis following cardiotoxin injury [28, 29]. Other host cell populations are unable to compensate for the loss of regenerative potential and muscle regeneration can only be rescued by replenishing the satellite cell pool [29]. In our study, $Pax7^+$ cells and small regenerating myofibers ($myosin^+$) were observed in the muscle scaffolds in close proximity to the remaining musculature, but were absent in the defect beyond 0.5 mm from the remaining muscle mass. In contrast, minced graft transplantation presented muscle fiber regeneration throughout the defect with associated satellite cell ($Pax7^+$) co-localization. Given the importance of satellite cells to muscle regeneration, the restricted satellite cell migration into the muscle scaffold appear to be a significant limitation to ECM-mediated muscle fiber regeneration.

Besides satellite cells, several other cell types such as bone marrow derived progenitors [30], pericytes [31, 32], mesoangioblasts [33], interstitial cells ($PW1^+/Pax7^-$) [34], perivascular stem cells [35], $CD133^+$ progenitors [36] have been shown to be myogenic. And, these alternative myogenic stem cells have been identified in remodeling ECMs [6, 36]. Overall, the contribution of these alternative myogenic cells towards regeneration is believed to be lower compared to satellite cells, but important never the less [30, 31]. We speculate that the therapeutic potential of these cells is limited to an even greater extent in a VML injury model, due to the stark absence of key myogenic cues from the lost myofiber remnants and satellite cells. In the highlighted study, $Sca\text{-}1^+$ cells migrated throughout muscle scaffold, and importantly in regions removed from the remaining muscle mass that were unpopulated by $Pax7^+$ cells. The presence of $Sca\text{-}1^+$ cells indicate that the muscle scaffold was conducive to stem and progenitor cells migration, but signify that their presence is not sufficient for *de novo* muscle fiber regeneration. Further studies are needed to assess if these potentially alternative myogenic cells require interaction with adult myofibers or activated satellite cells [37] to induce myogenesis.

Following injury, the type of the inflammatory response and the significance of transition from pro- to an anti-inflammatory phenotype is widely recognized as a critical component to regeneration. Through the phases of repair and regeneration, resident and recruited macrophages among other immune cells exhibit complex and hybrid phenotypes that are believed to be successively activated and timely subsided. While, pro-inflammatory activity is important for the migration and activation of myogenic precursor cells [38-40], an anti-inflammatory response stimulates muscle precursor cell differentiation and myotube formation [7, 38, 40, 41]. An extended and unrestricted pro-inflammatory (M1) macrophage response or an early and premature anti-inflammatory (M2) response has been shown to result in unsuccessful regenerative outcomes [19-23]. In our study, we generally characterized the inflammatory response of muscles repaired with muscle scaffolds and vital grafts at two weeks post-injury. The vital grafts supported a mixed up-regulation of pro- and anti-inflammatory markers *in vivo* that corresponded with improved regenerative outcomes. The overall inflammatory response appeared to be functionally attenuated in scaffold-repaired muscles, which corresponded with aberrant regeneration and fibrotic tissue deposition. Therefore, we speculate that both functionally active populations of pro- and anti-inflammatory cells (e.g., M1 and M2 macrophage phenotypes) are important for *de novo* regeneration of VML injured skeletal muscle. In support of this speculation, other studies have also demonstrated that coordinated efforts by both M1 and M2 macrophage phenotypes are necessary for scaffold vascularization [42] and remodeling [43]. A recent study by Novak *et al*., reported that macrophage populations present after muscle laceration injury, did not conform to the canonical M1/M2 classification. Furthermore, they demonstrated that activated donor M1 macrophages reduced fibrosis and enhanced myofiber regeneration but non-activated macrophages had no effect [44]. Collectively these data suggest that synchronized efforts of functionally active pro- and anti-inflammatory cells (e.g. M1 and M2 macrophage phenotypes) are required for effective regeneration.

Following recoverable injuries, factors released from injured muscle fibers induce an intricate spatiotemporal communication between the innate immune response and myogenic progenitor cell activity (i.e., primary satellite cells) that promotes repair and regeneration of the injured tissue. Pro-inflammatory M1 macrophages produce cytokines such as TNF-α and IL-6 that promote satellite cell activation and proliferation but not differentiation [45]. Anti-inflammatory M2 macrophages promote myoblast differentiation and fusion and myotube maturation by releasing cytokines such as interleukin (IL)-4 and IL-10 [38, 41]. In addition to secreted myogenic factors, human macrophages have also been shown to deliver anti-apoptotic signals to myogenic cells through direct cell-cell contact [46]. Evidence of two-way communication between myogenic cells and macrophages was demonstrated by our *in vitro* study in which secreted

factors from vital grafts were able to induce arginase expression (indicative of an M2-like phenotype) in macrophages. Therefore, disruption of either the inflammatory response or myogenic cell activity can significantly impact the regenerative outcomes. For instance, restricting monocyte/macrophage tissue invasion by disruption of CCL2/CCR2 signaling has been shown to impair regeneration and functional recovery [47, 48]. Likewise, ablation of satellite cells using an inducible Pax7 knockout model abrogated regeneration after toxin injury [28, 29]. The findings of our paper indicate that vital minced grafts comprised of myogenic cells in their native extracellular environment allow for interaction with the ensuing inflammatory response and promote effective muscle tissue regeneration. In contrast, transplantation of a devitalized scaffold results in a stark absence of satellite cells in distant regions of the defect and a diminished activity of pro- and anti-inflammatory cells. These observations present a deviation from the normal spatiotemporal events of muscle regeneration that is likely a major contributor to aberrant regenerative outcomes observed with ECMs. As the field continues to develop approaches to regenerate *de novo* muscle tissue after VML, ECMs and other scaffolds will likely be fundamental to these efforts. However, pursuant to improved regenerative outcomes with ECM-repair of VML is the development of strategies to further align the events of ECM remodeling with the intricate spatiotemporal events that underlie adult skeletal muscle regeneration.

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